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Spectrin-based skeleton in red blood cells and malaria

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Abstract

Malaria represents one of the most important selective factors affecting human populations. Several inherited diseases of red blood cells (RBCs) lead to resistance at the erythrocytic stage. Among patients who experienced hereditary elliptocytosis (HE) related to mutations of erythrocyte membranes proteins, molecular studies have shown the prevalence of particular spectrin mutations in patients from black ethnic extraction, leading one to question on the selection of new malaria-resistant genes.

Prospective epidemiological and molecular studies in West Africa have confirmed the prevalence (between 0.6 and 1.6%) of particular spectrin mutations related to HE. These studies have also revealed the frequency of α -spectrin chain polymorphisms, associated *in cis* with elliptocytogenic spectrin mutations and defining particular spectrin allele haplotypes. Culture studies of *P. falciparum* in elliptocytes bearing such elliptocytogenic alleles of spectrin showed that these alleles are supplementary genetic factors of malaria resistance *in vitro*.

It has not yet been shown that certain instances of spectrin mutations and/or polymorphisms constitute new factors of innate resistance to malaria *in vivo*. However, epidemiological survey of HE and parasite culture studies argue to explore more deeply the relationships between parasite and spectrin-based skeleton and to characterize molecular interactions between parasite ligands and particular spectrin chain domains.

Keywords: malaria, spectrin, hereditary elliptocytosis

Introduction

First identified at the intracellular surface of the erythrocyte membrane, spectrins (Sp) are now known to be the central component of the membrane skeleton, a ubiquitous and complex spectrin-actin scaffold located under the lipid bilayer of metazoan animal cells.

This network is responsible of red blood cell (RBC) shape and dynamic properties as clearly demonstrated by numerous mutations of its main components (particularly spectrin) associated with hereditary haemolytic anaemia such as hereditary elliptocytosis and spherocytosis [1, 2, 3*]. Spectrin (Sp) is giant extended flexible molecule composed of two non- identical subunits, α I (MW 280,000) and β I (MW 240,000) which intertwine to form heterodimers. Sp exists as elongated tetramers resulting from head-to-head self-association of heterodimers. Sp tetramers constitute the filaments of the lattice, the nodes of which are cross-linked by short actin filaments. This spectrin-based network is bound to various transmembrane proteins such as the integral protein band 3 (AE1) or glycophorins C and D through connecting proteins such as ankyrin and protein 4.1.

Malaria, caused particularly by *Plasmodium falciparum*, is the most serious and widespread parasitic disease of humans. Such a severe disease as malaria has exerted a powerful effect on human evolution and has operated a selection for resistance according to a number of host genotypes, leading to the appearance and persistence of a number of inherited diseases [4, 5, 6]. Many of these host genetic factors affect the RBCs, such as haemoglobinopathies (thalassemias, HbS, HbC, HbE), enzymopathies (glucose-6-phosphate deshydrogenase and pyruvate kinase deficiencies) and more recently diseases related to mutated membrane proteins, the most obvious being the Southeast Asia hereditary ovalocytosis associated to mutated protein band 3 [7, 8]. RBC polymorphisms such as Duffy

and Gerbich negative blood groups are also genetic factors which confer protection against malaria caused by *P vivax*. The human RBC is central not only for the parasite life cycle but also for the clinical symptoms which occur when parasites invade and multiply within RBCs. During intra-erythrocytic growth, the parasite extensively modifies the host erythrocyte membranes resulting in alterations of morphology (the cells become more spherical), increased membrane rigidity, reduced cell deformability and greater adhesive properties for the vascular endothelium and other blood cells [9]. These altered properties could result in part from the interaction of parasite proteins with spectrin-based skeleton. Following the example of haemoglobinopathies, molecular and epidemiological studies of hereditary haemolytic anaemia related to membrane defects lead one to question on the selection of new malaria-resistant genes uncoding some components of the spectrin-based skeleton.

Hereditary elliptocytosis related to spectrin defects and malaria

The first structural studies of spectrin molecule have been performed using limited digestion by trypsin. Tryptic resistant peptides (also called spectrin domains) can be separated and characterized by two dimensional gel electrophoresis (five domains for the spectrin α chain, from α I to α V, and four domains for the β chain, from β I to β IV). In most of the hereditary elliptocytosis (HE) related to a spectrin self-association defect, these tryptic patterns appeared modified with the presence of abnormal peptides, defined by their position and molecular masses. Among HE patients bearing abnormal spectrin tryptic patterns, we and others have rapidly observed that most of these patients have been of black ethnic extraction. Their spectrin tryptic patterns displayed most often an abnormal 46 or 65 kDa peptide variant, arising from the α I domain [1, 2, 3*, 10].

To confirm this possible prevalence of HE among black population, we have made prospective searches for HE in West Africa (Benin, Togo, Burkina Faso and Ivory Coast) [11,

12]. The diagnosis of elliptocytosis was established in a first step by the observation of a high percentage of characteristic regular and symmetric elliptic red blood cells (RBCs) after fixation in glutaraldehyde. The diagnosis was further confirmed by cytological studies of relatives and the discovery of a well defined molecular variant of spectrin. Among $\approx 5,000$ subjects, an incidence of elliptocytosis was found between 0.6 and 1.6%. All patients experienced mild, asymptomatic HE. Structural studies of spectrin have revealed the presence of two main tryptic phenotype: the $\text{Sp}\alpha^{I/65}$ and the $\text{Sp}\alpha^{I/46}$ variants at heterozygous state. Additional studies have shown that the $\text{Sp}\alpha^{I/65}$ variant presently found in West Africa underwent subsequent diffusion in North Africa, in Southern Italy as well as in West Indies and among African-American population [1, 2, 3*]. The $\text{Sp}\alpha^{I/65}$ variant was always related to the same mutation: the duplication of Leucine 154. When established, the $\text{Sp } \alpha\text{I}$ haplotype associated with this $\text{Sp}\alpha^{I/65}$ variant was always the same. The $\text{Sp}\alpha^{I/46}$ variant repeatedly found in Southern Benin and Togo among two ethnic groups (Fon and Yoruba) was associated with the same Leu260Pro mutation. Moreover, this mutation is most often associated *in cis* with the same spectrin αII and αIII domain polymorphisms, defining the $\text{Sp}\alpha^{\text{Nigerian}}$ allele [12]. The $\text{Sp}\alpha^{I/46}$ variant observed in other ethnic groups from West Africa (as Dendi people) was related to the Leu207Pro mutation. This mutation is borne by a low-expressed αI -spectrin allele (α^{LeIy} allele), defining the $\text{Sp}\alpha^{\text{St Louis}}$ allele [13].

Our molecular epidemiologic studies indicating that HE related to spectrin defects have an higher incidence in black ethnic populations than in Caucasian populations (up to 30 times) have raised the question whether these spectrin mutations provide some protection against malaria. However, none of the above spectrin mutations reaches such a high frequency as, for instance, the protein band 3 mutation accounting for ovalocytosis in Southeast Asia (up to 35% in particular areas) [7, 8] and well known to confer resistance to malaria invasion *in vitro*

[14]. It remains that the molecular characterization of spectrin defects in HE gives the opportunity to better study the relationship between the parasite and the erythrocyte skeleton.

Culture of *P.falciparum* in HE RBCs

We studied *in vitro* the invasion and the growth of *P.falciparum* in elliptocytes bearing haplotyped $\text{Sp}\alpha^{\text{I/65}}$ or $\text{Sp}\alpha^{\text{I/46}}$ variants. Highly synchronized cultures of *P.falciparum* performed in elliptocytes bearing a $\text{Sp}\alpha^{\text{I/46}}$ variant ($\text{Sp}\alpha^{\text{Nigerian}}$ allele) were followed during three erythrocyte life-cycles (Figure 1). The susceptibility of HE RBCs to invasion by parasites was examined using microscopic counting of infected elliptocytic RBCs and incorporation of [^3H]-hypoxanthine during maturation of ring-stage parasites to trophozoites. Growth abnormalities became apparent after the second cycle of parasite invasion (+96 hr) and more obvious after the third cycle (+144 hr). Similar reductions of merozoite invasion were observed in HE RBCs related to the $\text{Sp}\alpha^{\text{St Louis}}$ allele or to the $\text{Sp}\alpha^{\text{I/65}}$ variant (Figure 2). Considering all the results obtained at 96 hr with the three HE variants, the parasitized cells were reduced by 42% ($\pm 6\%$). The reduction of the [^3H]-hypoxanthine uptake was more pronounced (60%; $\pm 13\%$), arguing for a deficient parasite development, also illustrated by the increased percentage of trophozoite stage in the HE RBCs in contrast to schizont stage (Figure 2) and by ultrastructure studies of parasited elliptocytes. Transmission electron micrographs of immature schizonts in HE ($\text{Sp}\alpha^{\text{Nigerian}}$) RBCs elliptocytes showed accumulation of multilamellar structures in parasite cytoplasm (Figure 3). The same reduction of parasite invasion was observed when the second cycle is performed by reinvasion (1% infected cells; 2% hematocrit) of HE RBCs by merozoites that matured in the same HE RBCs. In contrast, the reinvasion of control RBCs by these conditioned merozoites remained normal (data not shown).

The growth inhibition seems to be unique, is that it appears after the second cycle and increases after the third one, suggesting that this growth abnormality must be cumulative. Schulman *et al.* have also reported that parasite growth in HE (Sp $\alpha^{I/65}$) RBCs started to differ from control RBCs after entering the second cycle and the diminution in parasite invasion became more significant during the subsequent cycles [15]. In this work, the authors have also shown that spherocytes with partial spectrin deficiency and elliptocytes completely deficient in protein 4.1 did not sustain normal parasite growth. Using a different invasion assay (percentage of ring-stage after 18 h of culture in a competitive invasion assay using fluorescein-labeled test cells), Facer reported a reduction of invasion into HE (Sp $\alpha^{I/65}$) RBCs between 24% and 55% [16]. In contrast, invasion into RBCs partially deficient in protein 4.1 was found either unchanged or increased. In the work reported by Chishti *et al.* [17], the invasion of merozoites into elliptocytes totally devoid of protein 4.1 was reduced by 60% right from the first cycle but, again, the reduction of merozoite invasion worsened after the second and third cycle. Moreover, the parasites derived from protein 4.1 (-) RBCs did not resume growth in normal RBCs in contrast to our results obtained with HE RBCs bearing Sp $\alpha^{I/65}$ or Sp $\alpha^{I/46}$ variants. Taking together, these results could indicate that the parasite was more weakened during its intracellular growth in the protein 4.1 (-) RBCs than during its live in HE RBCs related to a spectrin self-association defect.

From all these *in vitro* studies, it now appears to be well established that the parasite needs a normal erythrocyte skeleton to achieve complete and efficient invasion cycles. RBC membrane abnormalities, including either spectrin partial deficiency in hereditary spherocytosis, complete lack of protein 4.1 or spectrin self-association defect in hereditary elliptocytosis, impair the erythrocytic parasite cycles.

Most of the parasite proteins exported to the RBC membrane appear to be distributed around the erythrocyte skeleton and direct molecular interactions between host and parasite proteins

begin to be characterized [4]. The mature parasite-infected erythrocyte surface antigen (MESA) binds to protein 4.1 and the presence of protein 4.1 appears determinant in the traffic of MESA to the RBC membrane [18, 19]. The knob-associated histidine-rich protein (KAHRP), critically important for knob formation, binds to the α -spectrin chain and more precisely to the fourth tri-helical repeat. Moreover, this molecular interaction is required for assembly of KAHRP onto erythrocyte skeleton [20]. Finally, KAHRP interacts with the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which mediates the adhesion of parasitized RBC to the vascular endothelium. Interaction of spectrin with the major surface protein MSP-1 was also reported [21]. Erythrocyte skeleton proteins are targets for parasite proteases: falcipain-2 mediates cleavage of ankyrin and protein 4.1 [22, 23]; plasmepsin-II (an aspartic protease) degrades spectrin, actin and protein 4.1 [24] and protein band 3 is a target for Gp76 serine protease [25]. Other enzymes such as the *P. falciparum* long-chain acyl-CoA-synthetase (PfACS-1 and -3) presents experimental evidence for a high affinity interaction with ankyrin via the spectrin-binding region of ankyrin [26].

Spectrin α -chain polymorphisms and malaria

We have to keep in mind the possible role of spectrin α -chain polymorphisms that may or may not be associated with deleterious mutations causing HE. Two-dimensional gel electrophoresis of limited tryptic digestion of spectrin has revealed polymorphisms of the protein structure in the spectrin α II and α III domains [12, 27, 28]. Four patterns of α II domain peptides, based on their apparent molecular weight and isoelectric point have been identified (called type 1 to type 4). Amino acid sequencing and molecular studies [27, 28] have shown that these polymorphisms result from the combination of three points mutations at codons 701, 809, and 853, corresponding to amino-acids Arg, Ile and Thr in type 1; His, Val and Arg in type 2; His, Ile and Thr in type 3 and Arg, Val and Arg in type 4. In our

epidemiological study [12], Caucasian populations exhibit only the α II type 1 allele. In contrast, the α II-spectrin domain appears highly polymorphic in African population since the allele frequency for the α II type 2, 3, and 4 polymorphisms is increased to 40%. Compared to the sequence of the α II type 1 allele, one amino acid change was observed individually in one or the other of the three positions (codon 701, 809 or 853) in 32% of the alleles in the African population studied and the most frequent polymorphism, α II type 2 (24%), contains all three amino acid changes. Similar results have been reported in persons of African-American ancestry [27, 29]. The high prevalence of heterozygosity for these spectrin α II haplotypes (specially the α II-type 2) in individuals of African descent could suggest a selective advantage due to increased resistance of RBCs to malaria invasion. In the work published by DiPaolo *et al* [28]; the authors reported in the discussion that preliminary studies of malaria resistance of RBCs harbouring the α II domain haplotypes 2, 3, and 4 were not conclusive. Other α -spectrin chain mutations have been frequently found in individuals of African descent, such as the R1331I mutation accounting for the α III domain polymorphism [12] and the deleterious $\text{Sp}\alpha^{\text{St Claude}}$ allele. This allele is characterized by a splicing mutation that leads in part to a defective α -spectrin chain that cannot be assembled in the membrane. The patient homozygous for the $\text{Sp}\alpha^{\text{St Claude}}$ allele experienced a severe poikilocytic haemolytic anaemia. The unrelated heterozygous parents are clinically and biochemically normal and the $\text{Sp}\alpha^{\text{St Claude}}$ allele was identified in 3% of asymptomatic individuals from Benin [30].

Further studies are needed to clarify this issue because the location of these polymorphisms could involve α -spectrin domains implicated in unknown interactions with exported parasite proteins. Rather than *in vitro* studies of *P.falciparum* invasion in haplotyped RBCs, the knowledge of the complete genomic sequence of *P.falciparum* now allows the design of high-

throughput approaches to characterize direct interactions between parasite ligands and particular α -spectrin chain domains [31, 32].

Conclusions

It now appears that certain elliptocytogenic alleles of spectrin are supplementary genetic factors of malaria resistance *in vitro*, arguing to explore more deeply the relationships between parasite and spectrin-based skeleton. But it has not yet been shown that this potential resistance is expressed *in vivo* and that certain instances of spectrin mutations and/or polymorphisms constitute new factors of innate resistance to malaria.

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Figure legends

Figure 1:

Invasion and growth of *P.falciparum* (FcB₁/Columbia strain) into hereditary elliptocytosis RBCs (HE RBC) containing the Sp α ^{Nigerian} allele (L260P mutation associated with the α II-type 2 and α III-type 2 polymorphisms).

Highly synchronized and enriched cultures of *P.falciparum* were mixed with HE RBCs and control RBCs to attain a hematocrit of 2% and a parasitemia of 1%. The invasion and the growth of *P.falciparum* over three cycles (+48hr, +96hr and +144 hr) were studied using microscopic counting of infected RBCs and incorporation of [³H]-hypoxanthine.

Control RBC n°1 corresponds to a normal control Caucasian subject and the control RBC n°2 is a normal relative of the HE family (without any HE mutation or spectrin α -chain polymorphisms).

Figure 2:

Parasitemia observed after 2 cycles of *P.falciparum* invasion (FcB₁/Columbia strain) in control RBCs and in hereditary elliptocytosis RBCs (HE RBC).

Two haplotyped HE RBCs were studied: HE RBCs containing the Sp α ^{St Louis} allele (L207P mutation borne by the low-expressed α ^{Lely} allele) and HE RBCs containing the Sp α ^{L/65} variant related to the DupL154 mutation. Control RBC corresponds to a normal control Caucasian subject.

Parasitemia was determined by counting rings, trophozoites and schizonts on Giemsa-stained smears.

Figure 3:

Transmission electron micrograph of an immature schizont of *P.falciparum* (FcB₁/Columbia strain) in a HE RBC containing the Sp α ^{Nigerian} allele (courtesy of Dr Yves Boulard).

The presence of knobs on the RBC membrane is indicated by arrows. Notice the presence of multilamellar structures in the parasite cytoplasm (star).

N: nuclei; Pi, hemozoin (malarial pigment). Bar: 0.5 μ m.

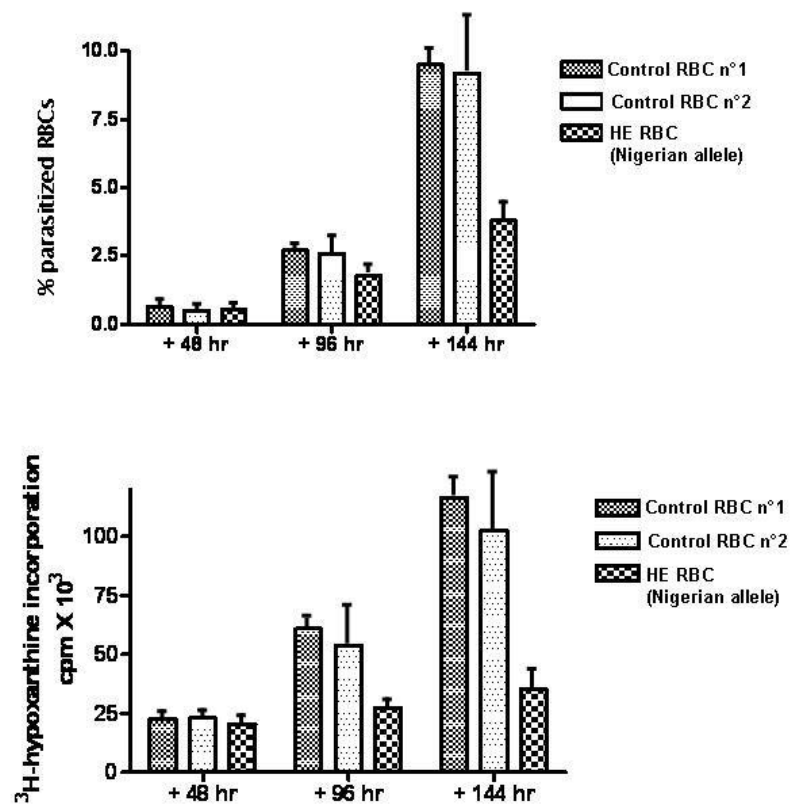


Figure 1

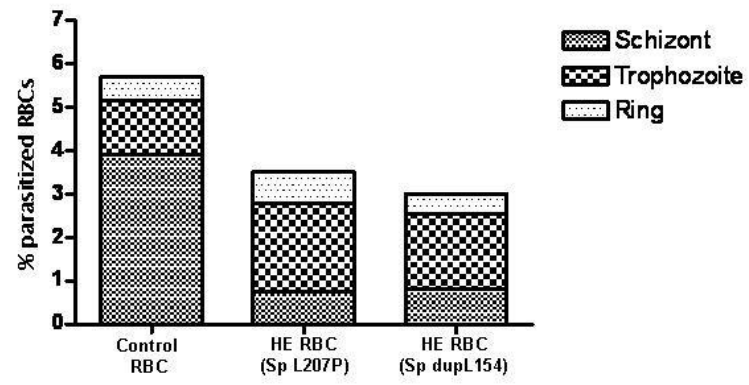


Figure 2

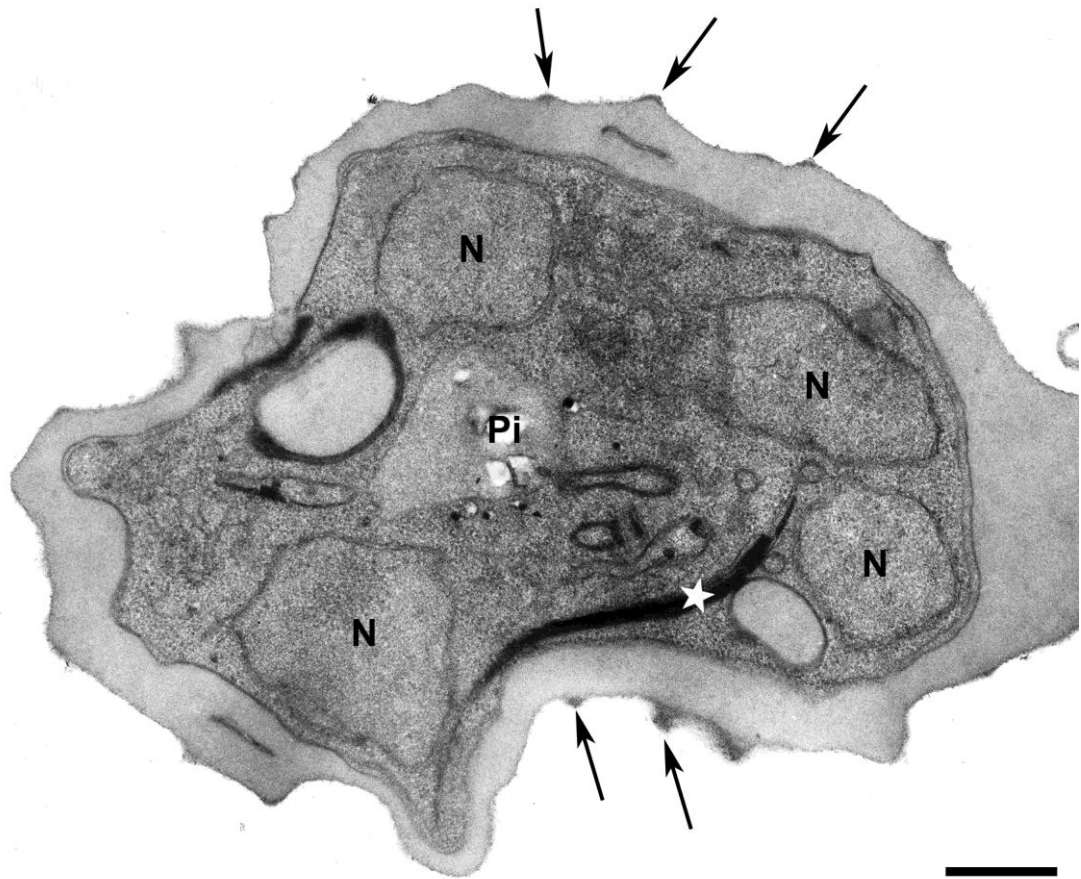


Figure 3